Cell, Vol. 83, 91–99, October 6, 1995, Copyright © 1995 by Cell Press

Evidence for Positional Differentiation of Prestalk Cells and for a Morphogenetic Gradient in Dictyostelium

Anne Early, Tomoaki Abe, and Jeffrey Williams
Medical Research Council
Laboratory for Molecular Cell Biology
and Department of Biology
University College London
London WC1E 6BT
England

Summary

We present evidence that Dictyostelium slug tip cells, the pstA cells, may arise by positional differentiation, but at a site remote from that which they will eventually occupy. When first detectable, the pstA cells form a peripheral ring surrounding the other prestalk cell subtype, the pstO cells, but subsequently move above the pstO cells to form the tip. Because pstA cell differentiation requires a 10-fold higher concentration of differention-inducing factor, the stalk cell inducer, the initial patterning seems likely to reflect the existence of a morphogenetic gradient. The subsequent redistribution of the two cell types is explicable by their different rates of chemotaxis to cyclic AMP. These results help reconcile the two apparently opposing views of pattern formation in Dictyostelium, that there is positional differentiation and that pattern formation occurs by cell sorting.

Introduction

To understand a morphogenetic process, it is of central importance to determine where differentiation is initiated. Differentiation in situ implies the existence of a locally acting morphogenetic signal, i.e., that some kind of positional information establishes pattern (Wolpert, 1971). If cells differentiate at apparently random sites and then move to their final resting place, a combination of a nonlocalized inducing signal and a cell-sorting mechanism is implied. Despite the apparent simplicity of Dictyostelium development, distinguishing these two possibilities has proven to be a long and tortuous process. It has been difficult because the organism seems to use elements of both systems of pattern formation. It employs a molecule with the properties expected of a morphogen to induce differentiation, but also uses intrinsic differences between cells to bias their fate and sorting to segregate them.

The Dictyostelium slug is constructed by the gathering together of up to 10^5 amoebae in response to pulsatile emissions of cyclic AMP (cAMP) from a signaling source at the center of the aggregate. The cells then pile atop one another to yield a mound-shaped structure called the tight aggregate. Subsequently, a tip arises at the apex of the mound that will become the tip of the migratory slug. The slug is patterned along its long axis, with prestalk cells occupying the front one fifth and prespore cells constituting the major cell type within the rear four fifths. Also, within the prespore region are scattered cells that show many of the properties of the prestalk cells and that are therefore called anterior-like cells (ALCs) (Sternfeld and David, 1981, 1982; Devine and Loomis, 1985).

The tip acts to organize the entire slug, so that if a tip is excised from one slug and grafted onto the side of another it will often subvert a fraction of the cells of the recipient and lead them away as a secondary slug (Raper, 1940; Rubin and Robertson, 1975; Durston, 1976). Understanding the initial stages of slug formation depends upon determining when, and where, the prestalk cells that make up the tip arise. Studies using two different markers of prestalk cell differentiation yielded an apparently unequivocal answer: prestalk cells arise at random positions within the aggregate and then accumulate at its apex (Datta et al., 1986; Williams et al., 1989).

Prestalk cell differentiation is induced by differentiation-inducing factor (DIF), a chlorinated hexaphenone that is produced during development and that is active at nanomolar concentrations (Kay and Jermyn, 1983; Morris et al., 1987; Town et al., 1976; Brookman et al., 1987). Since prestalk cells appeared to differentiate at random positions within the aggregate, DIF came to be viewed as an inducer of cellular differentiation rather than as a morphogen. Intrinsic differences between cells were usually invoked to explain the fact that neighboring cells within an aggregate could differentiate down alternate pathways. Such a belief had a strong basis for support, because a number of studies showed a correlation between position in the cell cycle and subsequent developmental fate (Weijer et al., 1984; McDonald and Durston, 1984; Gomer and Firtel, 1987; Ohmori and Maeda, 1987; Araki et al., 1994). This view shaped all recent descriptions of Dictyostelium pattern formation (Meinhardt, 1983; Schaap, 1986; Takeuchi, 1991; Weeks and Gross, 1991; Gross, 1994) but is, as we will show, only partially correct. The discovery of prestalk cell heterogeneity provides further insight into the process.

The slug synthesizes around itself a protein and cellulose-containing extracellular matrix called the slime sheath. The EcmA protein is a component of the sheath, where it plays a role in determining the shape of the slug during its formation (McRobbie et al., 1988). Expression of the *ecmA* gene is rapidly induced by, and dependent upon, DIF and occurs only in prestalk cells (Jermyn et al., 1987; Williams et al., 1987). There is a discontinuity in its level of expression: cells in the front half of the prestalk region express the gene somewhat more strongly than those in the rear half (Jermyn et al., 1989). Because of this difference in gene expression, the anterior prestalk cells are termed pstA cells and the posterior prestalk cells are termed pstO cells (Jermyn et al., 1989). The exact size of the tip has never been determined, because methods such as grafting lack the resolution to effect a precise localization. However, the tip must approximately equate with the pstA region.

Deletion analysis of the *ecmA* promoter has shown there

to be two separate regions that are able to direct expression in pstO cells: one region is independent of sequences directing expression in pstA cells, and a second region around the cap site is essential for expression in pstA cells (Early et al., 1993). Further analysis showed that the pstO and pstA cells are not static populations and introduced an additional complication concerning the ALCs. The smallest promoter fragment directing expression in pstO cells also directs expression in a subset of the ALCs (Early et al., 1993), and this subset was shown to constitute an interchanging population with the pstO cells (Abe et al., 1994).

We have identified a region of the *ecmA* promoter that directs expression only in pstA cells and compared the behavior of pstA cells with that of pstO cells. We show how it may now be possible to account for pattern formation, with a mechanism involving a morphogenetic gradient of DIF and a difference in chemotactic responsiveness of the two prestalk cell types.

Results

Identification of a Region of the *ecmA* Promoter That Directs Expression Specifically in PstA Cells

The intact promoter of the *ecmA* gene, coupled to the *lacZ* gene (the *ecmAO–lacZ* construct), directs expression in the pstA cells, the pstO cells, and the ALCs (Figures 1 and 2A). The *ecmA–lacZ* construct, which contains 529 nt of DNA sequence upstream of the cap site, shows strong expression in the approximate front one half of the prestalk zone, i.e., the pstA region (Figure 2B). There are a few scattered cells in the pstO region that express the construct, but even when there is a very high intensity of staining in the pstA region, the cells that surround the expressing cells in the pstO region remain completely unstained.

Fluorescence labeling of living cells has shown that there is a continuous forward movement as well as differentiation of pstO cells into pstA cells during slug migration (Abe et al., 1994). To determine whether the few, scattered *ecmA–lacZ*-expressing cells in the pstO region of the slug are pstO cells in the process of differentiating into pstA cells or are "misplaced" pstA cells, we performed immunostaining.

The *ecmO–c-myc* construct contains a Dictyostelium surface-expressed gene, bearing an epitope tag, that is expressed under the control of the *ecmO*-specific region of the promoter of the *ecmA* gene (Abe et al., 1994). Doubly transformed cells containing the *ecmA–lacZ* fusion gene and the *ecmO–c-myc* fusion gene were allowed to develop to the standing slug (first finger) stage and then fixed and stained (Figure 3A). The pstA cells stain red in this procedure, the pstO cells stain green, and coexpressing cells

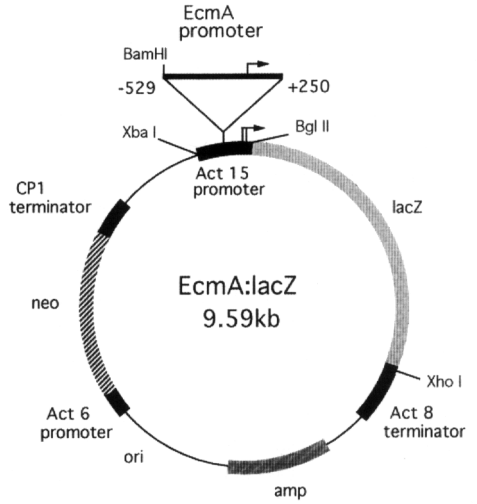

Figure 1. Structure of the *ecmA–lacZ* Construct, Which Directs Expression Specifically in PstA Cells

A fragment of the *ecmA* promoter, generated by polymerase chain reaction, was inserted into the unique BamHI cloning site of the Dictyostelium expression vector *A15ΔBam-gal* (Pears and Williams, 1988; Ceccarelli et al., 1991; construct O in Early et al., 1993). The actin 15 promoter sequences in this vector have been deleted to render them inactive, but retain sequences required for transcriptional initiation. The *ecmA* sequences comprise 529 nt upstream of the cap site, and 250 nt downstream, but lack an ATG (at +254 in the intact gene). The ATG is provided by the actin 15 gene, and it lies just upstream of the BglII site, through which the coding sequence is fused in frame to the *lacZ* gene of Escherichia coli. Primer extension analysis shows that although some transcripts initiate using the *ecmA* cap site, the majority utilize the two heterologous actin 15 start sites further downstream (data not shown). This indicates that *ecmA* initiation signals are dispensable for pstA expression.

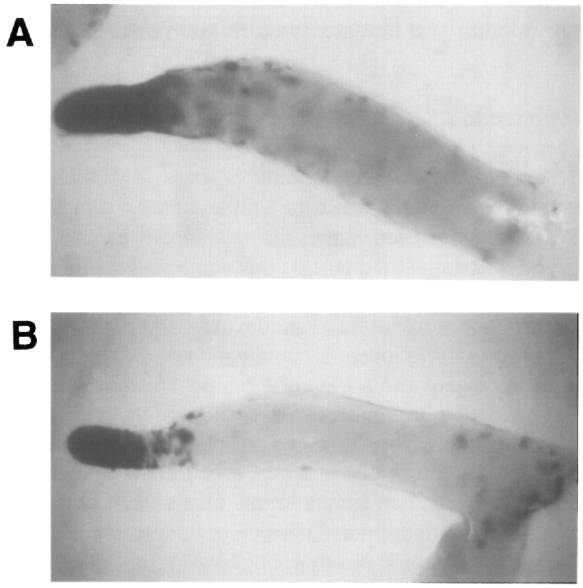

Figure 2. Comparison of the Expression of the *ecmA–lacZ* and *ecmAO–lacZ* Constructs in Newly Formed Slugs

Fixed whole mounts of developing structures were incubated in staining buffer for varying lengths of time to give comparable color intensity in the tip. After transformation and selection in 20 μg/ml G418, the cells used were cloned to maximize expression levels, but the staining patterns seen were unchanged compared with the original pooled populations (data not shown).

(A) Newly formed slug from cells transformed with *ecmAO–lacZ* (the intact promoter; Jermyn and Williams, 1991).

(B) Newly formed slug from cells transformed with *ecmA–lacZ* (pstA specific).

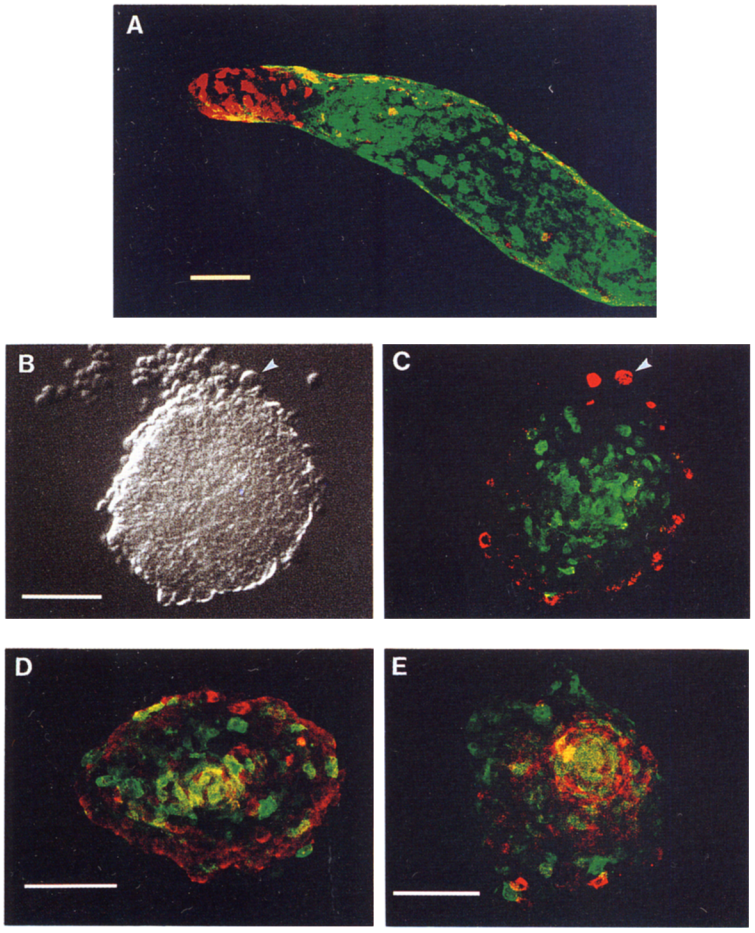

Figure 3. Analysis of Slug Formation and Structure Using Double Immunostaining of the *ecmA–lacZ* Marker and the PstO c-*myc* Marker

First finger (A); loose aggregate (B and C); tight aggregate (D); tipped aggregate (E). (A), (C), (D), and (E) are pseudocolor laser confocal images. Green represents the fluorescence of the pstO marker. Fluorescence from the pstA marker is shown in red. (B) Coexpressing cells appear yellow. Differential interference contrast (DIC) image of the aggregate pictured in (C). Scale bars represent 50 μm.

appear yellow. As expected (Abe et al., 1994; Early et al., 1993), almost all of the pstA cells are in the tip but many of the *ecmO*-expressing cells are located in the rear, prespore region. Thus, there is complete concordance between the *lacZ* and the immunofluorescence detection methods, confirming that the *ecmA–lacZ* construct is a specific marker for pstA cells. There are a few scattered yellow cells in the pstO region that are, we presume, pstO cells in the process of differentiating into pstA cells. (Note the faint yellow streaks around the periphery of the slug are a reflection background and do not derive from coexpressing cells.)

PstA and PstO Cells First Appear in Spatially Separate Regions of the Aggregate

Since the double-staining procedure proved to be a very sensitive detection method, we used it to investigate slug formation. It allowed detection of both pstA and pstO cells at the end of the loose aggregate stage, at a point when the last few cells were merging into the periphery (Figures 3B and 3C). Remarkably, there is an almost complete separation of the two cell types, the pstA cells forming an outer ring surrounding the pstO cells.

The fluorescence image (Figure 3C) is a composite of a series of confocal images focused at different depths. The individual images in the series show the pstA cells to be apposed to the substratum. This was expected, because at the very edge of a small aggregate such as this there is only one layer of cells. The pstO cells nearest the periphery are close to the substratum, but those near the center are present throughout the depth of the aggregate. It was not possible to study stages earlier than this, because these are partially disabled promoters and the analysis is at the limits of sensitivity of detection. Also, the loose aggregates are too fragile to withstand the methanol fixation necessary for the immune detection procedure. Hence, it was not possible to determine which cell type arises first.

Later in development, at the tight aggregate stage, there is still a heavy concentration of pstA cells in the periphery, but there are also strands of pstA cells within the aggregate (Figure 3D). When the tip begins to emerge, the movement of the pstA cells to the tip gives the impression of a spiral motion (Figure 3E), of the kind previously described for the prestalk-enriched markers and for cells in the prestalk region of the migrating slug (Esch and Firtel, 1991; Howard et al., 1992; Siegert and Weijer, 1992; Abe et al., 1994). Eventually, the pstA cells overtake the pstO cells and, by the standing slug stage (Figure 3A), they populate the entire tip with the pstO cells situated below them.

We interpret these movements as an influx of pstA cells from the periphery rather than transient expression in pe-

ripheral cells followed by de novo gene expression in cells at the center, because the β-galactosidase fusion protein in the pstA-specific construct is stable over a period of several hours (Detterbeck et al., 1994; H. MacWilliams, personal communication). Hence, it is necessary to posit the physical disappearance of pstA cells from the periphery of the aggregate rather than a turnover of β-galactosidase within a static population of cells.

PstO Cell Differentiation Requires a Lower Concentration of DIF than PstA Cell Differentiation

To compare the relative concentrations of DIF required to induce pstA and pstO cell differentiation, the *ecmA–lacZ* and *ecmO–lacZ* constructs were transformed into HMX44 (Kopachik et al., 1983; Morrison and Harwood, 1992). This is a Dictyostelium strain that is defective in the production of DIF but that remains DIF responsive. Using this strain, it is possible to perform induction at cell densities where, in the parental strain, endogenous DIF production would obscure the effects of exogenous DIF.

Expression of both the *ecmO–lacZ* and *ecmA–lacZ* fusion genes is dependent upon the presence of DIF, but as its concentration is reduced below 50 nM, the concentration that gives maximal induction, a marked difference becomes apparent (Figure 4). Half-maximal induction of pstA cell differentiation occurs at a DIF concentration of 5 nM, while half-maximal induction of pstO cell differentiation occurs at about 0.5 nM. We did not quantitate DIF degradation in these assays, so this difference could, in principle, reflect a difference in the time of exposure required to induce differentiation (see legend to Figure 4). However, this does not affect the basic conclusion: the DIF signaling pathway must be more highly stimulated if pstA cell differentiation is to occur.

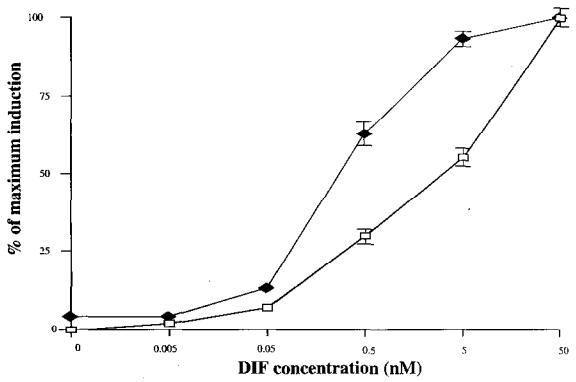

Figure 4. Comparison of the Levels of DIF Required to Give PstO- and PstA-Specific Differentiation in HMX44 Cells

The percentage of staining *ecmA–lacZ*-marked cells is indicated by open squares, and *ecmO–lacZ* cells are represented by closed diamonds. For each DIF concentration, approximately 300 cells were counted per experiment. The points shown on the graph are each an average of three experiments, and standard error bars are shown for those points at or above 10% maximal induction. The errors on the points below this were too small to present on this scale. The results are represented as percentages of the average total cell number staining with 50 nM DIF. The actual fractions of the population expressing at this concentration were the following: pstO construct, 70%; pstA construct, 25%. When the experiment was performed with 100 nM DIF, there was only a very slight increase in the numbers of stained cells seen (data not shown). We cannot be certain that the concentration of DIF at the end of the experiment will be as high as shown, because DIF induces the formation of DIFase (Insall et al., 1992). Because of the breakdown of DIF, it may be that pstA and pstO cell differentiation are occurring over different time scales. However, this does not affect the conclusion that more DIF is required to induce pstA cell differentiation than is required for pstO cell differentiation. The microscopic examination shows that this is an all-or-nothing response for an individual cell, suggesting that some kind of positive feedback loop may be involved.

Comparison of the Chemotactic Responsiveness of PstA and PstO Cells

The pstA cells occupy the tip of the slug, yet the data presented above suggest that they differentiate at the periphery of the aggregate. Prestalk cells are believed to achieve their apical position because they are more chemotactically responsive to cAMP than the prespore cells (Matsukama and Durston, 1979; Sternfeld and David, 1981; Wang and Schaap, 1985; Mee et al., 1986; Traynor et al., 1992) and because the tip is believed to act as a source of cAMP signaling (reviewed by Schaap, 1986). The generally held belief is that, during aggregation, the cAMP signaling center is composed of undifferentiated cells that are later replaced by prestalk cells that take over the signaling role. We therefore determined the relative responsiveness to cAMP of pstA cells, pstO cells, and prespore cells by in vivo labeling (Abe et al., 1994) and a chemotaxis assay.

The *ecmO–c-myc* construct directs surface expression of an epitope-tagged protein in pstO cells, and *pspA–c-myc* is an analogous construct that directs expression in prespore cells (Abe et al., 1994). These markers were compared with *ecmA–c-myc* (see Figure 5 for details of construction). Cells transformed with each of these constructs were allowed to develop to the first finger stage, disaggregated, and incubated with a monoclonal antibody specific for the c-Myc epitope. The antibody was fluorescently labeled with tetramethylrhodamine, so that cells expressing the surface marker could be identified (Figure 5). We have previously shown that cells retain their differentiated state after this treatment, because when they are allowed to reaggregate they return to the positions within the slug from whence they originally derived (Abe et al., 1994).

We determined responsiveness to cAMP by holding a pipette emitting cAMP close to a fluorescently labeled cell and recording the time it took to migrate to the pipette (Figure 5). The pstA cells moved more than twice as quickly as the pstO cells or the prespore cells. The pstO cells and the prespore cells move at very similar if not identical rates, the velocity difference we observe being too small to be statistically significant (Table 1). Thus, the higher rate of movement of the pstA cells may explain why they are able to move in from the periphery of the tight aggregate eventually to form the tip of the standing slug (Figures 3A, 3C, 3D, and 3E).

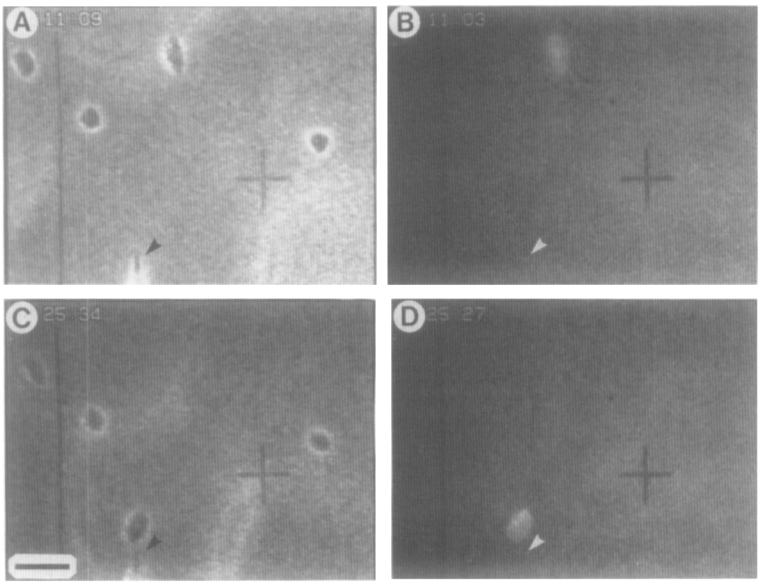

Figure 5. An Example of Analysis of Chemotactic Movement of Immunolabeled PstA Cells

(A) and (C) show phase-contrast images, while (B) and (D) show fluorescence images of the same fields. In this example, pstA cells were labeled with TRITC-conjugated antibodies (see Experimental Procedures). The cells were chemotactically stimulated with cAMP diffusing from the tip of a glass capillary. The position of the needle tip is indicated by an arrowhead in each picture. (A) and (B) were taken at the beginning of cAMP stimulation. (C) and (D) were taken 15 min later. The scale bar represents 50 μm.

Discussion

The existence at the mound and early tip stages of an outer skirt, from which prespore cells are excluded and wherein prestalk cells are enriched, is well established but has not been widely discussed (Takeuchi et al., 1978; Williams et al., 1989; Howard et al., 1992). The conclusion that emerges from the present study is that, at the end of the earlier, loose aggregate stage, the skirt region is entirely composed of pstA cells that encircle the pstO cells. We interpret this to mean that the very last cells to join the aggregate undergo positional differentiation to become pstA cells and then accelerate to reach the apex of the aggregate ahead of the pstO cells.

As always, when trying to establish the existence of a positional differentiation event, it is necessary to consider the alternative possibility: the cells differentiate elsewhere, at a stage before marker expression is detectable, and then move to the position where marker expression can be detected. This is a particularly acute problem in this case because there is abundant cell movement at this stage of development. Although we cannot entirely rule it out, one fact suggests that this is not likely to be true in this case. Expression of the pstA-specific marker in cells at, and just outside, the periphery of the aggregate (such as that arrowed in Figure 3B) gives the clear impression that cells are induced to become pstA cells as they approach the periphery of the aggregate. If a cell such as this had differentiated within the aggregate, it would have to have then moved out of the aggregate, i.e., away from the source of cAMP signaling. This runs contrary to all that is known about cell movement during aggregation, which is purely centripetal.

PstA and pstO cells both require DIF to differentiate, but pstA differentiation requires a 10-fold higher concentration than is required for pstO cell differentiation. We do not believe that this difference is likely to be due to impaired DIF inducibility resulting from the *ecmA* promoter having been pared down to yield a pstA-specific region. The amount of DIF required to induce pstA differentiation (half-maximal inducing concentration = 5 nM) is similar to that required for half-maximal induction of the intact *ecmA* promoter, i.e., 2 nM (M. J. Gaskell and J. W., unpublished data). Rather, it would appear that the pstO-specific region is particularly sensitive to DIF (half-maximal inducing concentration = 0.5 nM).

Since pstA cells are first detected at the periphery of the aggregate, it seems reasonable to suppose that DIF levels are highest in this region. The last cells to enter the mound will experience this high DIF concentration and will, therefore, differentiate as pstA cells (Figure 6). We have shown that pstA cells are more chemotactically responsive to cAMP than either pstO cells or prespore cells, and this would account for the subsequent reorganization

Table 1. Comparison of the Chemotactic Sensitivities of PstA, PstO, and Prespore Cells

PstA Cells	PstO Cells	Prespore Cells
12.0 μm/min	4.5 μm/min	3.4 μm/min

The *ecmO–c-myc* construct has been described previously (Abe et al. 1994), and the *ecmA–c-myc* construct was made by substituting the BglII–XhoI fragment of *ecmA–lacZ*, which contains the *lacZ* gene and actin 15 ATG, with a HindIII–XhoI fragment taken from a nonexpressing promoter deletion of *pspA–c-myc* (Early and Williams, 1989). The latter fragment contains 127 nt upstream of the *pspA* cap site and the entire c-*myc*-marked *pspA* gene (Abe et al. 1994). The HindIII and BglII ends were end filled prior to ligation. Cells expressing *ecmA–c-myc* (pstA cells), *ecmO–c-myc* (pstA cells), or *pspA–c-myc* (prespore cells) were detected by binding fluorescently labeled anti-c-Myc antibody (Abe et al., 1994), and their rate of movement to cAMP was determined (Figure 5). These are the average rates of migration of seven pstA cells, seven pstO cells, and ten prespore cells, respectively.

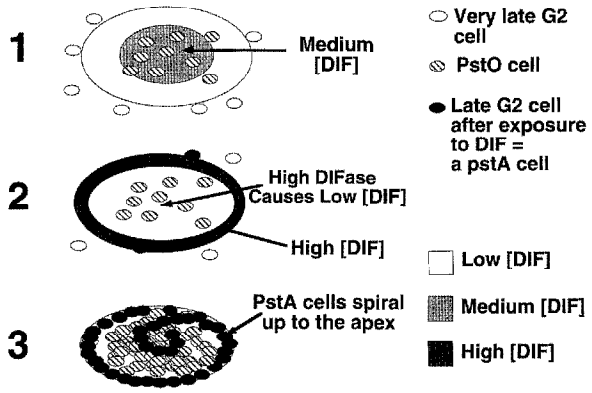

Figure 6. A Scheme for the Early Events in Slug Formation

(1) The unshaded plate represents a loose aggregate and is shown flat for the sake of clarity, although by this stage the center of the aggregate will be many cell diameters deep. Cells start to accumulate DIF at approximately this stage of development (Brookman et al., 1982). The actual identity of the cells producing DIF at this stage is not known, but in the migrating slug, DIF levels are highest in the prespore region (Brookman et al., 1987). At the stage represented, prespore cells are scattered within the aggregate but are excluded from the extreme periphery (Takeuchi et al., 1978; Williams et al., 1989). We have assumed there to be a uniform production of DIF by cells throughout the aggregate, although we show there to be a higher relative DIF concentration near the center. Development of the aggregate takes place under water, so the laws of diffusion and the dome-shaped stucture of the aggregate dictate that DIF will initially accumulate most quickly at the center. The pstO cells are shown as arising in the center, but this may not necessarily be the case. PstO cell differentiation could occur in situ in the center of the mound, they could differentiate at the periphery, and then move in ahead of the pstA cells, or alternatively, they could differentiate at random places within the aggregate and then move inward. The model only requires that the pstO cells come to occupy the central region of the aggregate, because this explains how a DIF sink comes to be formed at this position.

(2) The region of high relative DIF concentration is shown to have shifted to the periphery, where it is high enough to induce pstA cell differentiation. This distribution of DIF is based upon two assumptions.

The first is that the number of cells producing DIF increases over the period of tip formation. If, as seems most likely, the DIF-producing cells are the prespore cells (Brookman et al., 1987), this is reasonable because their number increases over this period (Takeuchi et al., 1978). It then becomes reasonable to suggest that the peripheral DIF concentration at this stage (stage 2) could be higher than the concentration of DIF at the center of the aggregate in stage 1 (panel 1).

The second is that the pstO cells at the center act as a DIF sink, because they produce DIFase. Again, it is important to emphasize that the aggregate develops under water so that DIF, a molelcule of low molecular weight, presumably diffuses freely into the surrounding environment. If, therefore, there is a DIF sink at the center, there should be a higher relative DIF concentration in cells at the periphery. There will also of course be simultaneous reverse diffusion (i.e., from the periphery to the inside), but this does not affect the model. The presumptive pstA cells perceive DIF synthesized from within the aggregate and are flanked by two regions where DIF is dissipated: the surrounding medium where DIF is lost by diffusion and the centrally located DIF sink. (Note that in the migrating slug DIFase is selectively localized in the tip, i.e., in the pstA cells [Kay et al., 1993], but this does not preclude the possibility that pstO cells at this early stage are producing DIFase. Using an unstable *lacZ* fusion gene construct (Detterbeck et al., 1994), we have show that expression of the *ecmA* gene via pstO-specific elements is turned off after a period of slug migration (A. E. and J. W., unpublished data), and this could equally well be true for the DIFase gene).

(3) Having experienced the high concentration of DIF at the periphery, the late G2 phase cells (Araki et al., 1994) become pstA cells and rapidly move up to the tip. The pstA cells may also accumulate DIFase, but we assume that they do not act as a DIF sink at the periphery, inhibiting further pstA cell differentiation, because they move rapidly away once they have differentiated. The pstA cells are shown to spiral upward because this is the impression that is obtained from looking at images such as Figure 3E, but the pstO cells could also adopt a spiral pattern of movement.

that leads to formation of the multilayered structure of the tipped aggregate.

We believe that the proposed ring of high DIF concentration at the periphery may be a consequence of the probable biphasic nature of prestalk cell differentiation and the existence of a DIF degradation pathway. The cells that produce DIF during slug formation have not been identified but, in assuming there to be a uniform distribution of DIF-producing cells during slug formation, the DIF concentration will initially be highest in the center of the mound. PstO cells differentiate at a low DIF concentration, and so they presumably differentiate before the pstA cells.

When a cell differentiates into a prestalk cell, it starts to produce DIF dechlorinase (DIFase), a cell-associated enzyme that inactivates DIF (Nayler et al., 1992; Insall et al., 1992; Kay et al., 1993). This establishes a negative feedback loop that limits the number of prestalk cells in an aggregate. The pstO cells in the center of the mound, therefore, would presumably act as a DIF "sink." Assuming it to be freely diffusible, DIF would then accumulate in highest concentration in the peripheral ring, and the last cells to enter the aggregate would be induced to become pstA cells. We assume that the pstA cells make DIFase but that they move away so rapidly that an effective DIF sink is not created at the periphery.

This model leaves a major question unanswered. If the concentration of DIF at the periphery is high enough to induce pstA cell differentiation, why is there also not simultaneous pstO differentiation at the periphery. Perhaps a specific inhibitor of pstO cell differentiation accumulates at the periphery, or it may be that high extracellular cAMP levels in the central region of the aggregate favor pstO differentiation. Further in vitro studies will be needed to resolve these possibilities.

Another question concerns the nature of the heterogeneity in the aggregating population that dictates that some cells will enter the aggregate early and become pstO cells while others will arrive late and become pstA cells. In this regard, one of the Dictyostelium cell cycle studies provides very strong support for the above model. It suggests that the relative time of arrival at the periphery of the aggregate depends upon cell cycle position at the time development starts and shows there to be exactly the inversion of relative position within the aggregate that we observe for pstA and pstO cells.

Dictyostelium cells spend most of the cell cycle in the G2 phase (Weijer et al., 1984). When cells expressing a stable marker (the *lacZ* gene under the control of a constitutive promoter) are synchronized in the cell cycle by a heat shock and mixed with nonsynchronized cells, they show one of two different behavior patterns depending

upon their cell cycle position at the time of starvation (Araki et al., 1994). Cells starved in middle to late G2 phase aggregate more rapidly than the synergizing partner cells but subsequently undergo a complete reversal of position and sort to the prespore zone. Conversely, cells starved in very late G2 phase are relatively slow to enter the aggregate but subsequently accelerate and move forward to populate the tip.

Thus, as was originally hypothesized by Maeda (1993), very late G2 phase cells show the pattern of movement that we have shown for pstA cells and have the same fate: these cells will form the tip. This behavior has only been observed for cells synchronized by heat shock, perhaps because the very late G2 phase cells that were studied by Araki et al. (1994) were overlooked in the other studies (McDonald, 1986; Wang et al., 1988; Weijer et al., 1984; Gomer and Firtel, 1987; Zimmerman and Weijer, 1993). There are fewer pstA cells than pstO cells within the slug (Figure 3A; unpublished data), and previous studies were presumably primarily focused on pstO cell differentiation.

This model (Figure 6) would explain why synchronized cells developing alone (i.e., without being synergized with an excess of nonsynchronized cells) show a normal prestalk:prespore ratio (Maeda et al., 1989). Even among synchronized cells, there will always be variation in chemotactic efficiency, so that the last cells to enter the aggregate will be exposed to the highest DIF levels and will therefore become pstA cells. On this view, cell cycle position does not determine cell fate, it merely helps to decide which cells are likely to encounter the positional signal: the ring of high DIF concentration at the periphery of the aggregate.

Experimental Procedures

Cell Culture and Transformation

Dictyostelium discoideum cells (AX-2 strain) were grown and transformed as described previously (Watts and Ashworth, 1970; Early and Williams, 1987). Clones were selected by plating amoebae in association with Klebsiella aerogenes and screening for β-galactosidase activity by an in situ detection method (Buhl et al., 1993). Cells of strain HMX44 (Morrison and Harwood, 1992), an axenic derivative of HM44, were transformed as for AX-2 cells, with the exception that selection was at 80 μg/ml G418, and analysis was of pooled populations. HMX44 cells were grown on plates both before and after transformation.

β-Galactosidase Staining of Slugs

Development was initiated by washing exponentially growing cells in KK2 (16.5 mM KH_2PO_4, 3.8 mM K_2HPO_4 [pH 6.2]). To encourage slug formation, the cells were plated at a density of 10^8/ml in thin streaks across 2% Bacto Agar (Difco) plates, perpendicular to a low level unidirectional light source. The plates were incubated at 22°C in a humid chamber for 16–18 hr. Slugs were fixed in 1% glutaraldehyde in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 2 mM $MgCl_2$) for 15 min and washed twice in Z buffer without fixative (Dingermann et al., 1989). Samples were then incubated in Z buffer containing 5 mM $K_3(Fe(CN)_6)$, 5 mM $K(Fe(CN)_6)$ and 1 mM X-Gal at 22°C until the appropriate degree of staining was obtained. The reactions were stopped by the addition of 1 mM phenylethyl-β-D-thiogalactoside, and the samples were mounted in Gelvatol before photography.

Double Labeling of Multicellular Structures with Fluorescent Antibodies

Cellular aggregates, developed on 2% nonnutrient agar plates, were transferred onto glass slides that had been coated with poly-L-lysine. Samples were fixed in absolute methanol for 5 min and then incubated for 24 hr at 4°C in a primary antibody solution containing both a mouse monoclonal anti-c-Myc antibody (9E10, Evan et al., 1985) and rabbit polyclonal anti-β-galactosidase antibody. Following several washes in phosphate-buffered saline (0.14 M NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 [pH 7.2]; three times for 5 min), the samples were incubated with a secondary antibody solution, containing FITC-conjugated anti-mouse IgG antibody and TRITC-conjugated anti-rabbit IgG antibody, for an additional 24 hr at 4°C. After repeated washing with PBS (three times for 5 min), the samples were sealed in Gelvatol. They were visualized with a laser confocal microscope (Bio-Rad, Model MRC1000). The optical sections were processed using NIH Image (NIH, v.1.57).

Induction of Marker Gene Expression by DIF

Subconfluent HMX44 cells, transformed with either the *ecmA–lacZ* marker or *ecmO–lacZ* (construct G in Early et al., 1993), were washed twice with KK2 and plated in submerged monolayer culture in stalk salts buffer (Kopachik et al., 1985). Incubation at a density of 2×10^5/ml was in the presence of 5 mM cAMP for the first 8 hr. The cells were then washed three times with stalk salts buffer before addition of buffer containing 5 mM cAMP, or additionally supplemented with DIF (a gift of R. Kay). After a further 18 hr, the cells were fixed in situ and stained overnight at 37°C using X-Gal as described above, and the percentage of staining cells was established.

Chemotaxis Test of In Vivo Labeled Cells

First fingers that had been developed on 2% nonnutrient agar plates were collected, resuspended in KNa_2 buffer (20 mM KH_2PO_4, Na_2HPO_4 [pH 7.0]), and mechanically dissociated by ten passages through a 25 gauge syringe needle. The cells were labeled with TRITC-conjugated 9E10 antibody (Abe et al., 1994). After labeling, the cells were resuspended in KNa_2 at a density of 1×10^6–2×10^6 cells/ml and placed in drops of 5–10 μl on glass slides previously coated with 1.0% nonnutrient agar. The samples were then incubated at 22°C for 10 min. Following incubation, thin sheets (less than 0.5 mm thick) of 1.0% nonnutrient agar were placed over the cells and excess liquid was removed (under these conditions, the cells are sandwiched between two agar sheets). The samples were viewed with an inverted fluorescence microscope (Zeiss, Model ICM405) equipped with an image-intensified CCD camera (Prostab Incorporated, Model HR604-MCP). During the course of the observation, cells were continuously stimulated with 100 μM cAMP diffusing from a fine glass needle tip. The movement of cells was recorded using a time-lapse video recorder (Matsushita, Model AG-6720A).

Acknowledgments

The initial stages of this study were supported by the Imperial Cancer Research Fund, and the work was completed with the support of the Wellcome Trust (Wellcome Program Grant 039899). We would like to thank Kei Inouye for invaluable advice on the chemotaxis assay and Yasuo Maeda, Bill Loomis, Harry MacWilliams, John Bonner, Adrian Harwood, Robert Insall, and Rick Firtel for their insightful and constructive comments on several earlier versions of this paper.

Received May 31, 1995; revised August 10, 1995.

References

Abe, T., Early, A., Siegert, F., Weijer, C., and Williams, J. (1994). Patterns of cell movement within the Dictyostelium slug revealed by cell type–specific, surface labeling of living cells. Cell *77*, 687–699.

Araki, T., Nakao, H., Takeuchi, I., and Maeda, Y. (1994). Cell-cycle dependent sorting in the development of *Dictyostelium* cells. Dev. Biol. *162*, 221–228.

Brookman, J. J., Town, C. D., Jermyn, K. A., and Kay, R. R. (1982). Developmental regulation of stalk cell differentiation-inducing factor in *Dictyostelium discoideum*. Dev. Biol. *91*, 191–196.

Brookman, J. J., Jermyn, K. A., and Kay, R. R. (1987). Nature and

distribution of the morphogen DIF in the *Dictyostelium* slug. Development *100*, 119–124.

Buhl, B., Fischer, K., and MacWilliams, H. K. (1993). Cell sorting within the prespore zone of *Dictyostelium discoideum*. Dev. Biol. *156*, 481–489.

Ceccarelli, A., Mahbubani, H., and Williams, J. G. (1991). Positively and negatively acting signals regulating stalk cell and anterior-like cell differentiation in Dictyostelium. Cell *65*, 983–989.

Datta, S., Gomer, R. H., and Firtel, R. A. (1986). Spatial and temporal regulation of a foreign gene by a prestalk-specific promotor in transformed *Dictyostelium discoideum*. Mol. Cell. Biol. *6*, 811–820.

Detterbeck, S., Morandini, P., Wetterauer, B., Bachmair, A., Fischer, K., and MacWilliams, H. K. (1994). The "prespore-like cells" of *Dictyostelium* have ceased to express a prespore gene: analysis using short-lived beta-galactosidases as reporters. Development *120*, 2847–2855.

Devine, K. M., and Loomis, W. F. (1985). Molecular characterization of anterior-like cells in *Dictyostelium discoideum*. Dev. Biol. *107*, 364–372.

Dingermann, T., Reindl, N., Werner, H., Hildebrandt, M., Nellen, W., Harwood, A., Williams, J., and Nerke, K. (1989). Optimization and *in situ* detection of *Escherichia coli* β-galactosidase expression in *Dictyostelium discoideum*. Gene *85*, 353–362.

Durston, A. J. (1976). Tip formation is regulated by an inhibitory gradient in the *Dictyostelium discoideum* slug. Nature *263*, 126–129.

Early, A. E., and Williams, J. G. (1987). Two vectors which facilitate gene manipulation and a simplified transformation procedure for *Dictyostelium discoideum*. Gene *59*, 99–106.

Early, A. E., and Williams, J. G. (1989). Identification of DNA sequences regulating the transcription of a *Dictyostelium* gene selectively expressed in prespore cells. Nucl. Acids Res. *17*, 6473–6484.

Early, A. E., Gaskell, M. J., Traynor, D., and Williams, J. G. (1993). Two distinct populations of prestalk cells within the tip of the migratory *Dictyostelium* slug with differing fates at culmination. Development *118*, 353–362.

Esch, R. K., and Firtel, R. A. (1991). cAMP and cell sorting control the spatial expression of a developmentally essential cell-type-specific *ras* gene in *Dictyostelium*. Genes Dev. *5*, 9–21.

Evan, G. I., Lewis, G. K., Ramsey, G., and Bishop, J. M. (1985). Isolation of monoclonal antibodies specific for the human c-myc proto-oncogene product. Mol. Cell. Biol. *5*, 3610–3616.

Gomer, R. H., and Firtel, R. A. (1987). Cell-autonomous determination of cell-type choice in *Dictyostelium* development by cell-cycle phase. Science *237*, 758–762.

Gross, J. D. (1994). Developmental decisions in *Dictyostelium discoideum*. Microbiol. Rev. *58*, 330–351.

Howard, P. K., Sefton, B. M., and Firtel, R. A. (1992). Analysis of a spatially regulated phosphotyrosine phosphatase identifies tyrosine phosphorylation as a key regulatory pathway in Dictyostelium. Cell *71*, 637–647.

Insall, R., Nayler, O., and Kay, R. R. (1992). DIF-1 induces its own breakdown in *Dictyostelium*. EMBO J. *11*, 2849–2854

Jermyn, K. A., and Williams, J. G. (1991). An analysis of culmination in *Dictyostelium* using prestalk and stalk-specific cell autonomous markers. Development *111*, 779–787.

Jermyn, K. A., Berks, M., Kay, R. R., and Williams, J. G. (1987). Two distinct classes of prestalk-enriched mRNA sequences in *Dictyostelium discoideum*. Development *100*, 745–755.

Jermyn, K. A., Duffy, K. T., and Williams, J. G. (1989). A new anatomy of the prestalk zone in *Dictyostelium*. Nature *340*, 144–146.

Kay, R. R., and Jermyn, K. A. (1983). A possible morphogen controlling differentiation in *Dictyostelium*. Nature *303*, 242–244.

Kay, R. R., Large, S., Traynor, D., and Nayler, O. (1993). A localized differentiation-inducing factor sink in the front of the *Dictyostelium* slug. Proc. Natl. Acad. Sci. USA *90*, 487–491.

Kopachik, W., Oohata, A., Dhokia, B., Brookman, J. J., and Kay, R. R. (1983). Dictyostelium mutants lacking DIF, a putative morphogen. Cell *33*, 397–403.

Kopachik, W., Dhokia, B., and Kay, R. R. (1985). Selective induction of stalk cell–specific proteins in *Dictyostelium*. Differentiation *28*, 209–216.

Maeda, Y. (1993). Pattern formation in a cell cycle dependent manner during the development of *Dictyostelium discoideum*. Dev. Growth Differ. *35*, 609–616.

Maeda, Y., Ohmori, T., Abe, T., Abe, F., and Amagai, A. (1989). Transition of starving *Dictyostelium* cells to differentiation phase at a particular position of the cell cycle. Differentiation *41*, 169–175.

Matsukama, S., and Durston, A. J. (1979). Chemotactic cell sorting in *Dictyostelium discoideum*. J. Embryol. Exp. Morphol. *50*, 243–251.

McDonald, S. A. (1986). Cell-cycle regulation of center initiation in *Dictyostelium discoideum*. Dev. Biol. *117*, 546–549.

McDonald, S. A., and Durston, A. J. (1984). The cell cycle and sorting behaviour in *Dictyostelium discoideum*. J. Cell Sci. *66*, 195–204.

McRobbie, S. J., Jermyn, K. A., Duffy, K., Blight, K., and Williams, J. G. (1988). Two DIF-inducible, prestalk-specific mRNAs of *Dictyostelium* encode extracellular matrix protein of the slug. Development *104*, 275–284.

Mee, J. D., Tortolo, D. M., and Coukell, M. B. (1986). Chemotaxis-associated properties of separated prestalk and prespore cells of *Dictyostelium discoideum*. Biochem. Cell Biol. *64*, 722–732.

Meinhardt, H. (1983). A model for the prestalk/prespore patterning in the slug of the slime mold *Dictyostelium discoideum*. Differentiation *24*, 191–202.

Morris, H. R., Taylor, G. W., Masento, M. S., Jermyn, K. A., and Kay, R. R. (1987). Chemical structure of the morphogen differentiation inducing factor from *Dictyostelium discoideum*. Nature *328*, 811–814.

Morrison, A., and Harwood, A. (1992). A simple method of generating axenic derivatives of *Dictyostelium* strains. Exp. Cell Res. *199*, 383–386.

Nayler, O., Insall, R., and Kay, R. R. (1992). Differentiation-inducing-factor dechlorinase, a novel cytosolic dechlorinating enzyme from *Dictyostelium discoideum*. Eur. J. Biochem. *208*, 531–536.

Ohmori, T., and Maeda, Y. (1987). The developmental fate of *Dictyostelium discoideum* cells depends greatly on the cell-cycle position at the onset of starvation. Cell Differ. *22*, 11–18.

Pears, C. J., and Williams, J. G. (1988). Multiple copies of a G-rich element upstream of a cAMP-inducible *Dictyostelium* gene are necessary but not sufficient for efficient gene expression. Nucl. Acids Res. *16*, 8467–8486.

Raper, K. B. (1940). Pseudoplasmodium formation and organization in *Dictyostelium discoideum*. J. Elisha Mitchell Sci. Soc. *56*, 241–282.

Rubin, J., and Robertson, A. (1975). The tip of the *Dictyostelium discoideum* pseudoplasmodium as an organizer. J. Embryol. Exp. Morphol. *33*, 227–241.

Schaap, P. (1986). Regulation of size and pattern in the cellular slime molds. Differentiation *33*, 1–16.

Siegert, F., and Weijer, C. J. (1992). Three-dimensional scroll waves organize *Dictyostelium* slugs. Proc. Natl. Acad. Sci. USA *89*, 6433–6437.

Sternfeld, J., and David, C. N. (1981). Cell sorting during pattern formation in *Dictyostelium*. Differentiation *20*, 10–21.

Sternfeld, J., and David, C. N. (1982). Fate and regulation of anterior-like cells in *Dictyostelium* slugs. Dev. Biol. *93*, 111–118.

Takeuchi, I. (1991). Cell sorting and pattern formation in *Dictyostelium discoideum*. In Cell–Cell Interactions in Early Development, J. Gerhart, ed. (New York: Wiley-Liss), pp. 249–259.

Takeuchi, I., Okamoto, K., Tasaka, M., and Takemoto, S. (1978). Regulation of cell differentiation in slime mold development. Bot. Mag. (special issue) *1*, 47–60.

Town, C. D., Gross, J. D., and Kay, R. R. (1976). Cell differentiation without morphogenesis in *Dictyostelium discoideum*. Nature *262*, 717–719.

Traynor, D., Kessin, R. H., and Williams, J. G. (1992). Chemotactic sorting to cAMP in the multicellular stages of *Dictyostelium* development. Proc. Natl. Acad. Sci. USA *89*, 8303–8307.

Wang, M., and Schaap, P. (1985). Correlations between tip dominance, prestalk/prespore pattern, and cAMP-relay efficiency in slugs of *Dictyostelium discoideum*. Differentiation *30*, 7–14.

Wang, M., Aerts, R. J., Spek, W., and Schaap, P. (1988). Cell cycle phase in *Dictyostelium discoideum* is correlated with the expression of cyclic AMP production, detection, and degradation. Dev. Biol. *125*, 410–416.

Watts, D. J., and Ashworth, J. M. (1970). Growth of myxamoebae of the cellular slime mould *Dictyostelium discoideum* in axenic culture. Biochem. J. *119*, 171–174.

Weeks, G., and Gross, J. D. (1991). Potential morphogens involved in pattern formation during *Dictyostelium* differentiation. Biochem. Cell Biol. *69*, 608–617.

Weijer, C. J., Duschl, G., and David, C. N. (1984). Dependence of cell-type proportioning and sorting on cell cycle phase in *Dictyostelium discoideum*. J. Cell Sci. *70*, 133–145.

Williams, J. G., Ceccarelli, A., McRobbie, S., Mahbubani, H., Kay, R. R., Early, A., Berks, M., and Jermyn, K. A. (1987). Direct induction of Dictyostelium prestalk gene expression by DIF provides evidence that DIF is a morphogen. Cell *49*, 185–192.

Williams, J. G., Duffy, K. T., Lane, D. P., McRobbie, S. J., Harwood, A. J., Traynor, D., Kay, R. R., and Jermyn, K. A. (1989). Origins of the prestalk-prespore pattern in Dictyostelium development. Cell *59*, 1157–1163.

Wolpert, L. (1971). Positional information and pattern formation. Curr. Topics Dev. Biol. *6*, 183–193.

Zimmerman, W., and Weijer, C. J. (1993) Analysis of cell cycle progression during development of *Dictyostelium* and its relationship to differentiation. Dev. Biol. *160*, 178–185.